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Research Article

Dissection of a novel molecular determinant mediating Golgi to *trans*-Golgi network transition

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Abstract. Two major functions of the Golgi apparatus (GA) are formation of complex glycans and sorting of proteins destined for various subcellular compartments or secretion. To fulfill these tasks proper localization of the accessory proteins within the different sub-compartments of the GA is crucial. Here we investigate structural determinants mediating transition of the two glycosyltransferases β-1,4-galactosyltransferase 1 (gal-T1) and the α-1,3-fucosyltransferase 6 (fuc-T6) from the *trans*-Golgi cisterna to the *trans*-Golgi network (TGN). Upon treatment with the ionophore monensin both glycosyltransferases are found in TGN-derived swollen vesicles, as determined by confocal fluorescence microscopy and density gradient fractionation. Both enzymes carry a signal consisting of the amino acids E_5P_6 in gal-T1 and D_2P_3 in fuc-T6 necessary for the transition of these glycosyltransferases from the *trans*-Golgi cisterna to the TGN, but not for their steady state localization in the *trans*-Golgi cisterna.

Keywords. Golgi Apparatus, sorting signal, trafficking, glycosyltransferases, monensin.

Introduction

The Golgi apparatus (GA) plays a major role in protein sorting and maturation. Secretory and membrane proteins destined for post-Golgi compartments pass through the GA where they are subjected to various structural changes such as glycosylation, acylation, sulfation and processing. The GA is the site where complex *N*-glycans are elongated and terminated. The enzymes involved in formation of complex glycans are the glycosyltransferases, which are all type II membrane proteins [1]. To fulfill their task they appear to be sequentially arranged along the *cis*- to *trans*-Golgi cisternal axis. Current evidence suggests co-localization of β-1,4-galactosyltransferase 1 (gal-T1; EC 2.4.1.38) with α-2,6-sialyltransferase 1 (sia-T1; EC 2.4.99.1) in *trans*-Golgi cisternae, while enzymes acting beforehand such as *N*-acetylglucosaminyltransferase I and mannosidase II are concentrated in medial cisternae [2]. How this sequential order of glycosyltransferases is established is not understood and several models have been proposed. In one model the length of the transmembrane domain determines the cisternal localization [3] yet it can not sufficiently explain the different dynamics of individual glycosyltransferases when the cells are treated with Golgi-disturbing agents such as monensin [4]. Evidence for another model involving Golgi matrix proteins for correct cisternal localization of glycosyltransferases has been proposed [5 – 7] that suggests that the cytoplasmic tail of glycosyltransferases can interact with Golgi matrix proteins and help mediating their correct localization.

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In the search for trafficking determinants for glycosyltransferases, in this study we investigated two Golgi glycosyltransferases, gal-T1, and the α-1,3-fucosyltransferase 6 (fuc-T6, EC not specified), both of which are sensitive to monensin treatment [4, 8, 9]. Translation initiation of gal-T1 can occur alternatively at either one of the two in-frame methionines resulting in a long form (24 amino acids) and a short form (12 amino acids) of the cytosolic tail. In addition, gal-T1 has been shown to be phosphorylated on serine residues within the cytosolic tail. Both features have been suggested to affect its trafficking behavior and/or monensin sensitivity [10, 11]. Treatment with this ionophore is used as a short-term read-out system to study Golgi to TGN translocation [4, 12]. Monensin, a carboxylic ionophore, leads to the efflux of protons from acidic post-Golgi organelles in exchange for sodium ions, followed by osmotic swelling of the endomembranes [13, 14] and the formation of TGN derived swollen vesicles [4, 12, 15], while the Golgi cisternae remain intact. These swollen vesicles contain gal-T1, fuc-T6 as well as the TGN marker TGN46 and CI-MPR [12], but are devoid of sia-T1 another *trans*-Golgi glycosyltransferase, which co-localizes with gal-T1 in untreated cells [4, 16, 17]. Short-term incubation with 2 μM monensin for 30 min leads to the formation of TGN-derived swollen vesicles, whereas prolonged treatment with 10 μM monensin leads to the accumulation of TGN46 and GPP130 in smaller peripheral structures [18].

Here we show qualitatively by confocal fluorescence microscopy as well as quantitatively by subcellular fractionation that the re-distribution of gal-T1 from the *trans*-cisterna to the TGN is mediated by a short amino acid signal consisting of a glutamic acid and a proline in its cytoplasmic tail. We extend this observation to fuc-T6, which carries a similar determinant on its cytoplasmic tail consisting of aspartic acid and proline. This [DE]P signal appears to be required for the anterograde transport of two glycosyltransferases from the *trans*-cisternae to the TGN.

Materials and methods

Materials. Chemicals were from AppliChem (Cheshire, CT) or Sigma-Aldrich (St. Louis, MO). Monensin was from Axxora Life Sciences Inc. (San Diego, CA). OptiPrep was obtained from Sigma-Aldrich. Enzymes used in molecular cloning were either from New England Biolabs (Ipswich, MA), Promega (Madison, WI), Roche Diagnostics (Basel, Switzerland), or Sigma-Aldrich. Nitrocellulose was from Whatman PLC (Florham Park, NJ); the ECL Western blotting reagents were from PerkinElmer Life Sciences (Waltham, MA). Polyethylenimine (PEI; linear, M_r 25 000) used for transfection was from Polysciences Inc. (Warrington, PA), Dulbecco's modified Eagle medium (DMEM) and bovine fetal calf serum (FCS) were from Invitrogen (Carlsbad, CA). Disposable plastic ware and cell culture dishes were from TPP (Trasadingen, Switzerland), VWR (Wien, Austria), Greiner Bio One (Monroe, NC) or Bioswisstec (Schaffhausen, Switzerland). DNA synthesis and oligonucleotide sequencing was performed by Microsynth GmBH (Galbach, Switzerland).

Antibodies. The monoclonal antibody to gal-T1 was gal-T1#2/36/118 [19], the monoclonal antibody to giantin (G1/133) was from Axxora Life Sciences Inc. and the monoclonal antibody to GFP was from Roche Diagnostics. The monoclonal goat anti-mouse antibody conjugated with Alexa 568 was from Molecular Probes (Eugene, OR) or conjugated with horseradish peroxidase (HRP) from GE Healthcare (Chalfont St. Giles, UK).

OptiPrep density gradient. The OptiPrep density gradient was performed according to the manufacturer's protocol (Axis Shield, protocol S36) and modified as described in Schaub et al. [4]. In brief, HepG2 cells were seeded on 10-cm plates and grown to 70 – 80 % confluency in DMEM containing 10 % FCS. The cells were then either incubated with 8 ml fresh DMEM + 10 % FCS or 8 ml fresh DMEM + 10 % FCS + 2 μM monensin for 30 min at 37°C. Thereafter the cells were transferred onto ice and washed twice with ice-cold PBS and once with homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4). The cells were then harvested in 2 ml homogenization buffer using a rubber policeman. To homogenize the cells, they were passed 15 times through a ball bearing homogenizer with a clearance of 16 μm and centrifuged at 800 *g* to remove unbroken cells and nuclei. Subsequently, 0.8 ml homogenate was loaded onto an OptiPrep step gradient containing 2.5 – 30 % iodixanol (final volume of 9.2 ml: 0.8 ml 2.5 %; 1.3 ml each of 5 %, 7.5 % and 10 %; 0.8 ml 12.5 %; 1.3 ml 15 %; and 0.8 ml each of 17.5 %, 20 % and 30 %). The gradient was centrifuged for 2.5 h at 200 000 g_{av} in the Sorvall TH-641 swing-out rotor. Finally, ten fractions of 1 ml were collected from the bottom by perforating the tube with a G21 needle, and proteins were precipitated using trichloroacetic acid (TCA) as described below, then analyzed by Western blotting using an anti-GFP antibody.

SDS-PAGE, immunoblotting and quantification. Using the Laemmli [20] system the proteins were separated on 10 % SDS-polyacrylamide minigels

(Bio-Rad, Hercules, CA) and then transferred to nitrocellulose membranes using the method described by Towbin et al. [21]. Immunoblotting was performed as described previously [22]. In short, membranes were washed in phosphate-buffered saline (PBS) and then blocked for 30 min using 3% milk in PBS containing 0.01% thimerosal. Anti-GFP antibody was applied overnight at 4°C in PBS containing 3% milk. After washing in PBS the secondary antibody conjugated with HRP was applied for 1 h in PBS containing 3% milk and, after final washing, the chemiluminescence was recorded using the quantitative densitometer from Raytest (Straubenhardt, Germany). Subsequently, the recorded data were analyzed using the Aida Software (Straubenhardt, Germany).

Confocal immunofluorescence imaging. For immunofluorescence imaging, cells were grown on coverslips to a density of ~50% and either incubated with fresh growth media with or without 2 μM monensin for 30 min. The cells were then washed in PBS and fixed using 3% paraformaldehyde (PFA) in PBS. The cells were washed with PBS-glycine (20 mM glycine in PBS) and solubilized using PBS-glycine-saponin (PBS containing 0.1% saponin and 20 mM glycine). Subsequently, the cells were incubated in the first antibody diluted in PBS-saponin (PBS containing 0.1% saponin), washed four times in PBS-saponin, incubated with the secondary antibody conjugated with the fluorophore in PBS-saponin, washed again four times in PBS-saponin and finally mounted on a glass slide using Prolong Gold antifade (Invitrogen, Carlsbad, CA). The pictures were recorded using a Leica SP5 confocal laser scanning microscope and analyzed using the Imaris software from Bitplane (Zurich, Switzerland).

TCA precipitation. For TCA precipitation, the collected 1-ml gradient fractions were supplemented with 190 μl 0.15% deoxycholate, 20 μg bovine serum albumin (BSA) and mixed thoroughly. Then 200 μl 100% TCA was added and the mixture was incubated for 1 h to overnight on a rotating shaker at 4°C. The proteins were then collected by centrifugation (Max speed for 30 min at 4°C) and washed twice using cold acetone (stored at – 20°C). Finally, the pellet was resuspended in protein gel-loading buffer.

Generation of mutant gal-T1 and fuc-T6 constructs. To generate the novel gal-T1-GFP mutants, vector containing gal-T1-GFP [4] was digested using *Xba*I and *Sfa*NI. Complementary oligos, encoding for the mutant cytoplasmic tail with overhangs compatible with the *Xba*I/*Sfa*NI-digested vector containing gal-T1-GFP, were annealed by heating them to 94°C and then slowly cooled in ligation buffer. The annealed oligos were then directly ligated into the *Xba*I/*Sfa*NI open vector containing gal-T1-GFP. The fuc-T6-RFP construct used as template was a gift from L. Borsig. First the pFX vector was prepared by removing the native *Bgl*II site from pcDNA3.1 (Invitrogen) and gal-T1-GFP [4] was sub-cloned into this vector. The cytoplasmic tail, transmembrane domain and stem region of fuc-T6 were amplified by PCR using oligonucleotides containing either the *Xba*I site and wild-type or mutant tail sequence for the sense oligo or the *Bgl*II restriction site in the antisense oligo. Finally, the PCR product was sub-cloned into *Xba*I/*Bgl*II open pFX containing GFP. Further details are given in the supplementary material.

Tissue culture and transfection. HepG2 cells were grown in DMEM + 10% FCS at 37°C in a 5% CO_2 atmosphere. Transfection was performed according to the protocol from Polyplus-Transfection (Illkirch, France). In brief, HepG2 cells were grown in six-well plates to a confluency of ~25%. DNA (4.4 μg) and PEI (15 μg) were mixed and then added to the cells in 1 ml of culture media. At 24 h after transfection the cells were washed with fresh culture media. At 48 h post transfection the cells were trypsinized and seeded on 10-cm plates at dilutions 1:10, 1:100 and 1:1000. Finally, at 3 – 4 weeks post transfection clonal colonies were picked and expanded for analysis.

Results

E5P6 motif in the cytoplasmic tail of gal-T1-GFP is required for redistribution of gal-T1-GFP chimera into TGN-derived swollen vesicles. Gal-T1 and sia-T1 share a similar domain structure and localization to the *trans*-cisternae of the GA [16], and they catalyze subsequent steps in glycosylation. Both enzymes are readily redistributed to the endoplasmic reticulum (ER) upon brefeldin A treatment [4] showing that they are not localized to the TGN but to the Golgi cisternae. However, using the carboxylic ionophore monensin as short-term read out system, we could show that sia-T1 remains in the perinuclear region together with other Golgi markers such as giantin, while gal-T1 separated from the GA and redistributed to TGN-derived swollen vesicles [4]. This displacement is referred to here as "monensin sensitivity". We also demonstrated that replacement of the catalytic domain of gal-T1 with green fluorescent protein (GFP) neither altered its subcellular localization nor influenced its response to monensin. Using GFP chimerae we showed that the first 13 amino acids of

Figure 1. Overview of GFP-conjugated glycosyltransferases β-1, 4-galactosyltransferase 1 (gal-T1-GFP), α-1,3-fucosyltransferase 6 (fuc-T6-GFP) and their mutants.

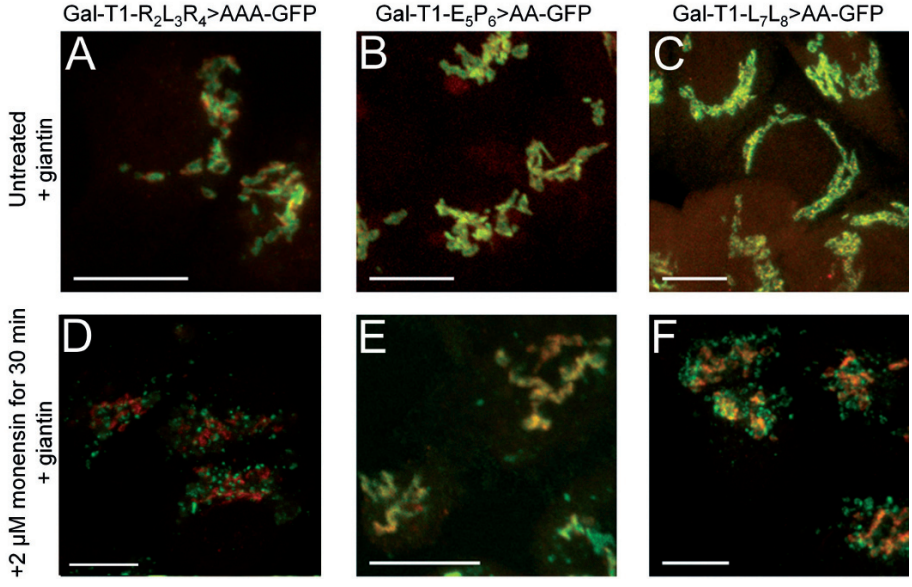

Figure 2. Fluorescence of HepG2 cells stably transfected with gal-T1-$R_2L_3R_4$>AAA-GFP, gal-T1-E_5P_6>AA-GFP and gal-T1-L_7L_6>AA-GFP (all in green) and immunolabeled with a monoclonal antibody against giantin (red). All three mutants co-localize with giantin (*A*, *B* and *C*, respectively) in untreated cells. Upon treatment with 2 µM monensin for 30 min both gal-T1-$R_2L_3R_4$>AAA-GFP (*D*) and gal-T1-L_7L_6>AA-GFP (*F*) separate from giantin and are found in swollen vesicles (arrows). In contrast the gal-T1-E_5P_6>AA-GFP mutant is not redistributed to swollen vesicles and remains co-localized with giantin (*E*). Bar 10 µm

the cytoplasmic tail of gal-T1 (unique to the long form of gal-T1) are necessary for redistribution of gal-T1 to TGN-derived swollen vesicles. In addition, we demonstrated that the two potential phosphorylation sites S_9 and S_{11} are not necessary for monensin sensitivity of gal-T1. We therefore generated three novel alanine mutants scanning for the crucial amino acids involved in monensin-induced redistribution of gal-T1 (Fig. 1). All three mutant chimerae co-localized with endogenous giantin in the same structures of the perinuclear region in untreated cells as shown by confocal fluorescence microscopy (Fig. 2A – C), indicating a correct localization of the mutant fusion protein. The overlap between gal-T1 and its derivatives with giantin is significant, albeit not complete as giantin staining appears more on the periphery of the GA enclosing gal-T1 staining [23]. Treatment with brefeldin A redistributed all the GFP chimerae to the ER, further substantiating their Golgi localization and excluding a steady-state accumulation in the TGN (data not shown). The two mutants gal-T1-$R_2L_3R_4$>AAA-GFP and gal-T1-L_7L_8>AA-GFP behaved as wild-type gal-T1-GFP in monensin-treated cells and moved to swollen vesicles (Fig. 2D, F). In contrast, gal-T1-E_5P_6>AA-GFP showed little reaction to monensin treatment and remained in the perinuclear region co-localized with endogenous giantin (Fig. 2E). To test the contribution of E_5 and P_6 to monensin sensitivity of gal-T1, we created additional mutants replacing the two amino acids individually by alanine (Fig. 1). Both single alanine mutants (gal-T1-E_5>A-GFP and gal-T1-P_6>A-GFP) co-localized to the GA together with giantin in untreated cells (Fig. 3A, B) and were redistributed to the ER upon treatment with brefeldin A (data not shown). Upon treatment with monensin their localization remained unchanged in the GA, where they were co-localized with giantin (Fig. 3C, D), showing that E_5 and P_6 are crucial for monensin-induced redistribution of gal-T1 into TGN-derived swollen vesicles.

To confirm these results by a quantitative approach, cell homogenates were fractionated on an OptiPrep

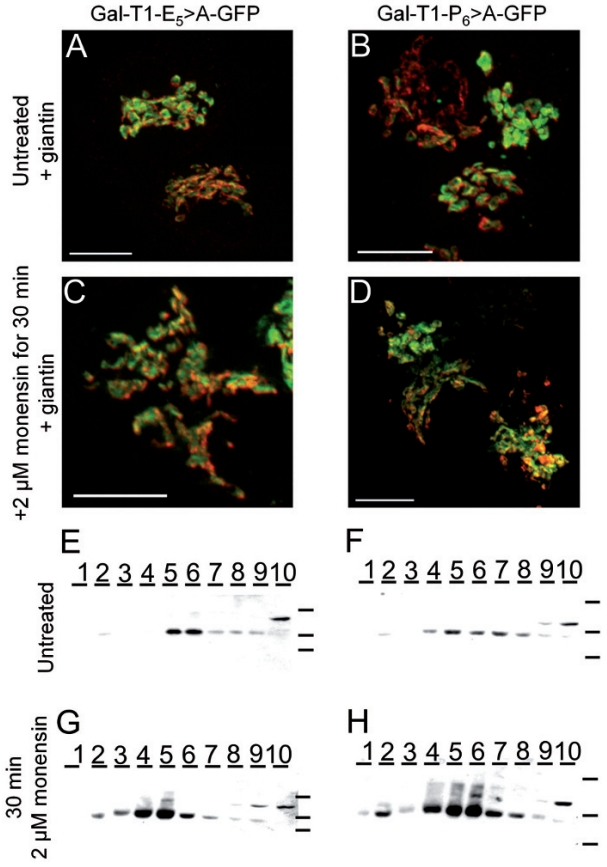

Figure 3. (*A – D*) Fluorescence of HepG2 cells stably transfected with gal-T1-E_5>A-GFP and gal-T1-P_6>A-GFP (green) and immunolabeled with a monoclonal antibody against giantin (red). Both mutants co-localize with giantin (A and B, respectively) in untreated cells. Upon treatment with 2 µM monensin for 30 min neither of the mutants is displaced and they remain co-localized with giantin. Bar 10 µm. (E – H) OptiPrep density gradient of HepG2 cells stably transfected with gal-T1-E_5>A-GFP or gal-T1-P_6>A-GFP. The density of membranes found in fraction 1 is highest and decreases towards fraction 10. Both gal-T1-E_5>A-GFP and gal-T1-P_6>A-GFP culminate in fractions 5 and 6 in untreated cells (*E* and *F*). No shift towards lighter fractions can be observed upon treatment with 2 µM monensin for 30 min (*G* and *H*), indicating monensin insensitivity of the two mutants.

density gradient to separate swollen vesicles from the GA. In this gradient dense membranes such as the ER accumulate in the lower fractions and the GA located to fractions 4 – 6. The swollen vesicles floated towards the lightest fractions 7 – 10. Both gal-T1-$R_2L_3R_4$>AAA-GFP and gal-T1-L_7L_8>AA-GFP showed a clear shift towards the lighter fractions 7 – 10, representing 40.7 % and 16.9 % of total immunoreactive protein loaded on the gradient, respectively, when comparing monensin-treated cells with untreated cells, showing the redistribution of the two chimerae to the swollen vesicles (Table 1). In contrast, only a slight shift of the gal-T1-E_5P_6>AA-GFP mutant was found, representing 13.7 % in the lighter fractions in

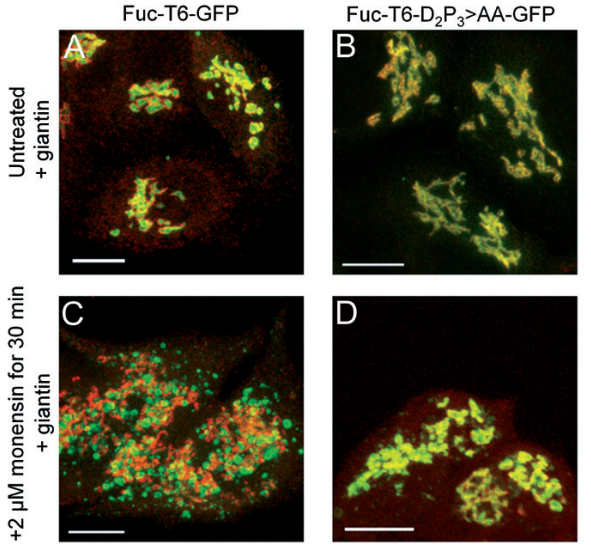

Figure 4. (*A – D*) Fluorescence of HepG2 cells stably transfected with fuc-T6-GFP and fuc-T6-D_2P_3>AA-GFP (green) and immunolabeled with a monoclonal antibody against giantin (red). Both mutants co-localize with giantin (*A* and *B*, respectively) in untreated cells. Upon treatment with 2 µM monensin for 30 min fuc-T6-GFP separates from giantin and is found in swollen vesicles (*C*), whereas the mutant fuc-T6-D_2P_3>AA-GFP remains co-localized with giantin in the Golgi apparatus (GA) (*D*). Bar 10 µm

monensin-treated cells, which was only 4.3 % higher than the value in untreated cells. These data are in accordance with the results observed by fluorescence microscopy (Table 1).

In gradients of untreated cells, gal-T1-E_5>A-GFP and gal-T1-P_6>A-GFP showed a clear peak in fractions 5 – 6 and 5 – 7, respectively (Fig. 3E, F). Neither mutant redistributed towards the lighter fractions upon monensin treatment since gal-T1-E_5>A-GFP peaked in fractions 4 – 6 and gal-T1-P_6>A-GFP in fractions 5 – 6 (Fig. 3G, H). Quantification of the single mutants showed that gal-T1-E_5>A-GFP and gal-T1-P_6>A-GFP was found in fractions 7 – 10 to the same extent in monensin-treated cells as in untreated cells with a difference of only 2.3 % and 1.5 %, respectively, which was also in accordance with the results obtained by fluorescence microscopy.

D_2P_3 motif in the cytoplasmic tail of fuc-T6 is crucial for the monensin sensitivity of fuc-T6. To test whether the motif found in gal-T1 is unique or a more general signal we investigated fuc-T6, a glycosyltransferase previously shown to be monensin sensitive [8]. Fuc-T6 co-localizes with giantin and gal-T1 in the GA (Figs 4A, 5A). As expected, upon treatment with monensin, fuc-T6 was redistributed to TGN-derived swollen vesicles, where it was co-localized with gal-T1 but not giantin (Figs 5C, 4C). Since the sequence D_2P_3 in the cytoplasmic tail of

Table 1. Quantification of monensin sensitivity.

	% of total immunoreactive protein loaded on gradient found in fractions 7 – 10		
	Control	Monensin treated	
gal-T1-GFP	20.2 ± 8.5	58.6 ± 11.4*	
sia-T1-GFP	21.3 ± 6.2	22.3 ± 7.5*	
gal-T1-$R_2L_3R_4$>AAA-GFP	8.6 ± 7.3	49.3 ± 8.8	Fig. 2A, D
gal-T1-E_5P_6>AA-GFP	9.4 ± 5.4	13.7 ± 4.8	Fig. 2B, E
gal-T1-L_7L_8>AA-GFP	11.2 ± 6.6	28.1 ± 7.2	Fig. 2C, F
gal-T1-E_5>A-GFP	17.2 ± 4.7	19.5 ± 5.5	Fig. 3E, G
gal-T1-P_6>A-GFP	19.6 ± 4.0	21.1 ± 7.7	Fig. 3F, H
fuc-T6-GFP	8.0 ± 2.4	36.5 ± 4.1	Fig. 5E, G
fuc-T6-D_2P_3>AA-GFP	11.4 ± 3.0	9.3 ± 2.5	Fig. 5F, H
gal-T1-E_5>D-GFP	13.3 ± 4.8	24.9 ± 6.1	Fig. 6E, G
fuc-T6-D_2>E-GFP	9.7 ± 1.9	22.0 ± 5.9	Fig. 6F, H

n= 4 – 6
* From Schaub et al. [4]

fuc-T6 is similar to the E_5P_6 motif of gal-T1 we generated the fuc-T6-D_2P_3>AA-GFP mutant to test if these two amino acids exert the same function in fuc-T6 as E_5P_6 in gal-T1 (Fig. 1). In untreated cells the fuc-T6-D_2P_3>AA-GFP co-localized together with giantin and endogenous gal-T1 in the GA (Figs 4B, 5B). Like the gal-T1-E_5P_6>AA-GFP mutant, the fuc-T6-D_2P_3>AA-GFP mutant was unaffected by treatment with monensin and remained in the GA together with giantin (Fig. 4D), while endogenous gal-T1 was separated from the mutant and segregated into swollen vesicles (Fig. 5D).

To confirm these findings, segregation of fuc-T6 was also analyzed by cell fractionation using OptiPrep density gradient as described above. Both fuc-T6-GFP and fuc-T6-D_2P_3>AA-GFP showed a strong peak in fractions 4 – 5 in gradients obtained from untreated cells (Fig. 5E, F). The fuc-T6-GFP chimerae showed a substantial shift towards the lighter fractions 7 – 10 in cells treated with monensin (28.5 % more than for untreated cells) (Fig. 5G). Unlike the wild-type fuc-T6-GFP chimerae, the mutant fuc-T6-D_2P_3>AA-GFP chimerae did not shift towards the lighter fractions and remained in the peak fractions 5 – 6. There was no difference in the amount of fuc-T6-D_2P_3>AA-GFP when comparing monensin-treated cells to untreated cells (Fig. 5H).

These quantitative results from the OptiPrep density gradients confirmed that the motif D_2P_3 is analogous to E_5P_6 of gal-T1 in determining monensin-induced redistribution of fuc-T6 into TGN-derived swollen vesicles. Taken together these results suggest that the determinant [ED]P exerts a function mediating Golgi to TGN transition.

Replacement of E_5 with aspartic acid in gal-T1-GFP and of D_2 with glutamic acid in fuc-T6-GFP strongly reduces monensin sensitivity of the two mutants. We also replaced E_5 with aspartic acid in gal-T1-GFP and D_2 with glutamic acid in fuc-T6-GFP (Fig. 1) to test whether the two signals from gal-T1 and fuc-T6 can substitute for each other. In control cells, both gal-T1-E_5>D-GFP and fuc-T6-D_2>E-GFP co-localized with giantin in the GA (Fig. 6A, B). Upon treatment with monensin only a small amount of gal-T1-E_5>D-GFP was found in swollen vesicles, while most of the mutant remained co-localized with giantin in the GA (Fig. 6C). Like gal-T1-E_5>D-GFP, only a small fraction of the fuc-T6-D_2>E-GFP mutant was found in swollen vesicles after monensin treatment, while the majority remained in the GA where it co-localized with giantin (Fig. 6D).

These findings were further validated using the OptiPrep density gradient. In gradients of untreated cells both mutant chimerae were strongly enriched in fractions 4 – 5 (Fig. 6E, F). Upon treatment with monensin, only 11.6 % more of the gal-T1-E_5>D-GFP were found in fractions 7 – 10 when compared to untreated cells showing a clear reduction of redistribution of the mutant into swollen vesicles (Fig. 6G). Accordingly, the fuc-T6-D_2>E-GFP only showed 12.3 % more floating towards fractions 7 – 10 upon treatment with monensin compared to untreated cells (Fig. 6H). Thus, both gal-T1-E_5>D-GFP and fuc-T6-

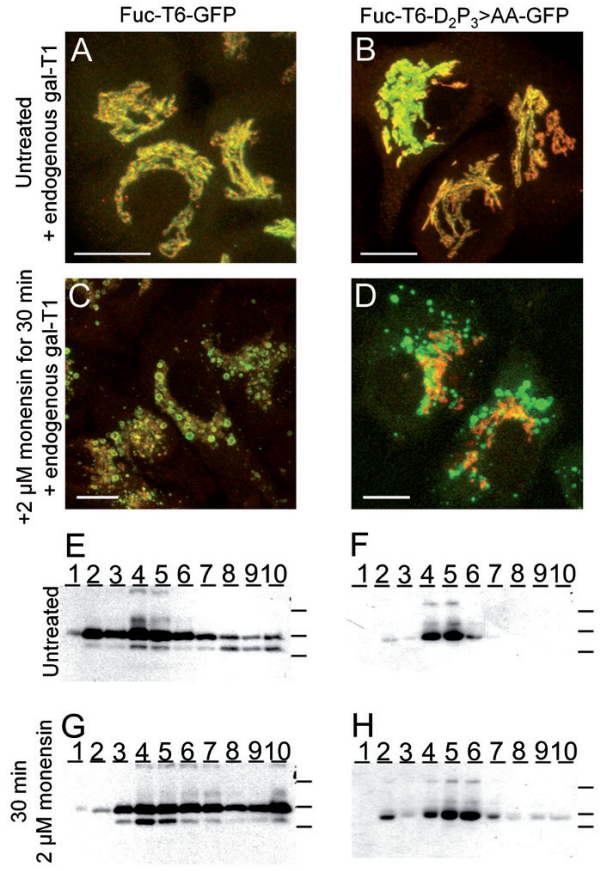

Figure 5. (*A – D*) Fluorescence of HepG2 cells stably transfected with fuc-T6-GFP and fuc-T6-D_2P_3>AA-GFP (green) and immunolabeled with a monoclonal antibody against gal-T1 (red). Both mutants co-localize with endogenous gal-T1 (*A* and *B*, respectively) in untreated cells. Upon treatment with 2 µM monensin for 30 min fuc-T6-GFP is redistributed to swollen vesicles where it co-localizes together with endogenous gal-T1 (*C*). Unlike fuc-T6-GFP, the mutant fuc-T6-D_2P_3>AA-GFP is insensitive to monensin treatment and remains associated with the GA (*D*). Bar 10 µm. (*E – H*) OptiPrep density gradient of HepG2 cells stably transfected with fuc-T6-GFP or fuc-T6-D_2P_3>AA-GFP. Both fuc-T6-GFP and fuc-T6-D_2P_3>AA-GFP culminate in fractions 4 and 5 in untreated cells (*E* and *F*). A clear shift towards lighter fractions can be observed upon treatment with 2 µM monensin for 30 min for fuc-T6-GFP (*G*), while the mutant fuc-T6-D_2P_3>AA-GFP remains concentrated in fractions 4 and 5 (*H*).

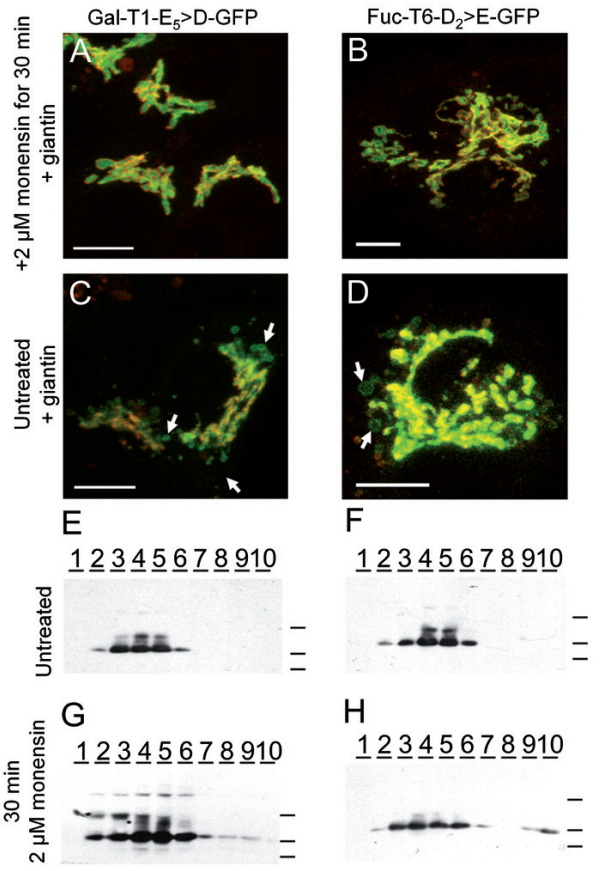

Figure 6. (*A – D*) Fluorescence of HepG2 cells stably transfected with gal-T1-E_5>D-GFP and fuc-T6-D_2>E-GFP (green) and immunolabeled with a monoclonal antibody against giantin (red). Both mutants co-localize with giantin (A and B, respectively) in untreated cells. Upon treatment with 2 µM monensin for 30 min most of gal-T1-E_5>D-GFP and fuc-T6-D_2>E-GFP remain associated with the GA where they co-localize with giantin, and only a small fraction separates from the GA and is found in swollen vesicles (*C* and *D*, arrows). Bar 10 µm. (*E – H*) OptiPrep density gradient of HepG2 cells stably transfected with gal-T1-E_5>D-GFP or fuc-T6-D_2>E-GFP. Both gal-T1-E_5>D-GFP and fuc-T6-D_2>E-GFP culminate in fractions 4 and 5 in untreated cells (*E* and *F*). A slight shift towards lighter fractions can be observed upon treatment with 2 µM monensin for 30 min (*G* and *H*), indicating a strongly reduced monensin sensitivity of the two mutants.

D_2>E-GFP exhibit a reduced redistribution to swollen vesicles compared with their wild-type counterpart. This reduction indicates a requirement for additional structural features in the respective cytoplasmic tails.

Discussion

The discovery of a trafficking motif operating between the GA and the TGN addresses important aspects on the transition from the GA to the TGN. In fact, virtually nothing is known about this step. While a consensus on mechanisms of intra-Golgi transport centers on the cisternal maturation model [24, 25], the transition from Golgi to TGN is still an enigma. In our view, *trans*-Golgi cisternae and the TGN are, for three main reasons, distinct, stable entities: first, brefeldin A induces retrograde flow of Golgi membrane proteins to the ER on one hand [26], and centers TGN membrane proteins around the mitochondrial organization center on the other hand [27], and thus defines a division between both organelles. Second, the TGN may be delineated by its standard marker protein TGN38/46 whose steady-state distribution excludes the *trans*-Golgi cisterna as defined by gal-T1 localization [28]. Finally, the transition of proteins from the

trans-Golgi cisterna to the TGN takes 4 min, as measured by Mironov et al. [29], which is similar to the time measured for the transition from the ER to the *cis*-Golgi (also 4 min) and much slower than the transfer through the individual Golgi cisternae (2 min), indicating a more complex trafficking step for the transition from *trans*-Golgi cisterna to TGN. These dynamics support the assumption of a discrete transition step from the GA to the TGN. Here we used monensin as a short-term read out system to further analyze this trafficking step. How this step is mediated is unclear at present. However, our data suggest that in addition to the present model of cisternal maturation and retrograde trafficking of resident proteins, further mechanisms are required to explain the transition from the *trans*-Golgi cisterna to the TGN. Both gal-T1-E_5P_6>AA-GFP and fuc-T6-D_2P_3>AA-GFP are retained in the *trans*-Golgi cisterna at steady state by retention or recycling. The [DE]P motif would therefore provide additional functionality by either retaining the two glycosyltransferases in the maturing cisterna for delivery to the TGN by preventing their recycling to earlier Golgi cisternae; or by sorting them into transport intermediates capable of delivering them from the *trans*-Golgi cisterna to the TGN.

The presence of the [DE]P motif in addition to the retention/recycling signal would require some sort of switch to alternate between them. The experiments performed by Hathaway et al. [30] and Reynolds et al. [31] indicated the possibility that phosphorylation might be involved for this step. However, we could not find any evidence in support of the involvement of phosphorylation for monensin sensitivity of gal-T1 [4]. Furthermore, fuc-T6 has no potential phosphorylation site in its cytoplasmic tail excluding a direct regulation of fuc-T6 by phosphorylation.

A possible reason for the presence of the signal mediating Golgi to TGN transport may be the facilitation of a so-called ectopic localization [32] or displacement to a post-Golgi processing site. Both gal-T1 and fuc-T6 are known to occur in a soluble form in body fluids *in vivo* [33, 34]; the processing site and the corresponding proteases remain to be determined. As already pointed out by Pfeffer [35] in a recent review and by Tu et al. [36], there seems to be no unique mechanism governing trafficking of glycosyltransferases. This somewhat sobering conclusion is also highlighted by the role attributed to the conserved oligomeric Golgi complexes (COGs) believed to mediate retrograde transport of Golgi cisternal elements. In this context, several Golgi components were examined for their COG dependency: here again, some of them were shown to depend on COGs, termed GEARs [37], others not. More recently, Vps74p and TMF/ARA180 have been suggested to directly mediate Golgi localization of glycosyltransferases by interaction with their cytoplasmic domain, although it is unclear at which stage they operate [7, 38]. However, it has been suggested that VPS74p is involved in retrograde trafficking of glycosyltransferases [36].

Table 2. Membrane proteins carrying a [DE]P signal close to the end of their cytoplasmic tail.

Protein class	Number containing a DP or EP motif	
	Type II	Type I
Glycosyltransferases	3	
Trafficking related proteins	6	
Proteases	5	
Carriers and transporter	27	1
Others	32	12
Total	82	13

A thorough bio-informatics analysis was performed to search for membrane proteins containing the [ED]P motif close to the cytoplasmic terminus (Tables 2, 3 and S1). To identify proteins with transmembrane domains and their topology, the method described by Kall et al. [39] was used and the database was subsequently scanned for the motif. The resulting proteins were sorted by membrane topology. Of the 101 type II proteins (see Table S1), those that were known to be localized to the ER, nucleus, mitochondria and cytosol were excluded. The remaining 82 proteins were further attributed to relevant groups (Table 2). Out of the 304 type I proteins, only 13 contained the motif at the C terminus in the cytosol. The most prominent group of proteins containing the [ED]P motif comprises the transporters and carriers (Table S1). Since plasma membrane localization of those proteins is tightly regulated (reviewed in Bradbury and Bridges [40]) one could speculate that the [DE]P motif might mediate sorting into a specialized, regulated, exocytic pathway. Another prominent group are the proteases (Table 3). Proteases are known to be involved in regulation of transporters (reviewed in Hughey et al. [41]), and therefore it would make sense that the proteases are sorted into the same pathway. In addition, one of the proteases could potentially be responsible for the processing of gal-T1 and/or fuc-T6 as described above. Finally, six proteins involved in trafficking that contain the [DE]P motif were identified (Table 3). The most prominent of them, the SNARE GOSR2, has been described as mediator for the trafficking from *cis*-/

Table 3. Proteins involved in trafficking, post-Golgi proteases and an additional glycosyltransferase.

Glycosyltransferases		
SIA8E	Alpha-2,8-sialyltransferase 8E	D_5P_6
B4GAT	Beta-1,4-galactosyltransferase 1	E_5P_6
FUT6	Alpha-1,3-fucosyltransferase 6	D_2P_3
Trafficking		
GOSR2	Golgi SNAP receptor complex member 2	D_2P_3
SCAMP1	Secretory carrier-associated membrane protein	D_3P_4
STX6	Syntaxin-6	D_5P_6
STX8	Syntaxin-8	D_5P_6
STX10	Syntaxin-10	D_4P_5
VAPB	Vesicle-associated membrane protein-associated protein B/C	D_3P_4
Proteases		
DPP6αα	Dipeptidyl aminopeptidase-like protein 6	E_6P_7
HPN	Serine protease hepsin	D_2P_3
LNPEP	Leucyl-cysteinyl aminopeptidase	E_2P_3
TMPRSS4	Transmembrane protease, serine 4	D_4P_5
TMPRSS9	Transmembrane protease, serine 9	E_2P_3

medial-Golgi to *trans*-Golgi/TGN [42] as well as ER to *cis*-Golgi [43]. It would be interesting to test whether any of these trafficking-related proteins are required for the monensin sensitivity of gal-T1 and fuc-T6. This could help to elucidate the mechanism by which proteins transit from the *trans*-Golgi to the TGN.

Concerning glycosyltransferases, little is known with respect to their specific molecular determinants for their retention or retrieval. Apart from the kin recognition hypothesis that may explain retention of *N*-acetylglucosaminyltransferase I and mannosidase II in medial-Golgi cisternae [44], the length of the transmembrane domain in the case of sia-T1 [3] and the cytoplasmic tail of glycolipid forming glycosyltransferases [6, 7, 38] were shown to have an influence for their localization. For the sorting event between the trans-Golgi cisterna and the TGN, individual transmembrane domains or luminal domains of either gal-T1 or sia-T1 could be excluded as sorting determinants, shown by domain shuffling experiments [4]. Strong evidence for signal operating in signal-mediated anterograde trafficking was provided in the work of Reynolds et al. [31]. Using a truncated version of α-1,3-mannosyltransferase (Mnn1p) they showed that its retention to the *trans*-Golgi cisterna is mediated by its luminal domain. Upon knockdown of the mitogen-activated protein kinase Hog1p, this truncated mutant of Mnn1p was secreted, suggesting that Hog1p regulates either the retention or retrieval of Mnn1p. However, in wild-type cells the truncated Mnn1p is not secreted, indicating that retrieval is not required for its Golgi localization. The implication of a luminal domain as a site for a retention signal addresses a mechanism differing from the one described here and that might be similar to the mechanism required for the retention of soluble Golgi proteins [45].

In conclusion, it appears that no direct experimental evidence is available yet concerning the mechanism for *trans*-Golgi to TGN transition. Here we show that using monensin as a read-out system to trap gal-T1 and fuc-T6 in TGN-derived swollen vesicles, Golgi to TGN transition involves (a) a specific set of *trans*-Golgi enzymes, i.e., gal-T1 and fuc-T6, and (b) that the signal mediating this transition consists of two critical amino acids [ED]P located in the cytoplasmic tail of these enzymes.

Electronic supplementary material. Supplementary material is available in the online version of this article at springerlink.com (DOI 10.1007/s00018-008-8446-y) and is accessible for authorized users.

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